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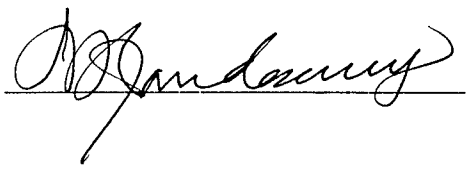

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13. ABSTRACT ***(Maximum 200 Words)***

The goal of this proposal is to test the hypothesis that loss of expression of a novel protein contributes to tumor growth, invasion and metastases, and its expression suppresses these biological events. We have identified, isolated and partially characterized a 55 kDa nuclear matrix protein from human breast tumor cells (hence forth referred to as nmt55). This novel protein is expressed in estrogen receptor positive (ER+) tumors but its expression is greatly reduced in ER- tumors. Decreased expression of nmt55 correlated strongly with tumor size ($p<0.03$) and loss of ER and PR ($p<0.001$). As tumor size increased, nmt55 protein expression decreased. Because increased tumor size is associated with metastases, we postulate that decreased nmt55 expression is associated with molecular and cellular changes linked to cellular differentiation leading to loss of ER expression, and development of hormone-independent tumor growth, invasion and metastases. We are currently investigating the function of nmt55 using biochemical and molecular biology approaches. The information derived from these studies will help determine the potential role of nmt55 as a marker of tumor progression and metastases. Further, these studies may provide critical information needed for early detection of potentially metastatic tumors, improve diagnosis and prognosis and aid in developing strategies for therapeutic management and care of breast cancer patients.

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Matthew Pavao 7-30-01

PI - Signature Date

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Potential Role of a Novel Nuclear Matrix Protein (nmt55) as a Tumor Marker in Human Breast Cancer Invasion and Metastases.

Introduction:

The development of breast cancer is thought to be a multi-stage process [1]. The progression of this disease is associated with cellular and molecular changes. Thus, initiation and progression may be related to loss of chromosomal material and ultimately specific gene function(s). Some of these cellular and molecular changes may be accompanied with tumor cell acquisition of metastatic potential. There is an urgent need for identification of node-negative patients whose tumors have metastatic potential. Several tumor markers have been used in assessing tumor changes linked to poor prognosis. These include loss of estrogen receptor (ER) and progesterone receptor (PR) expression [2], high blood vessel count (angiogenesis) [3], amplification of erbB2/HER2/neu gene [4] and decreased activity of nm23 gene [5]. None of these markers alone, however, predict with complete reliability, which node-negative patients will likely relapse. We have found that primary human breast tumors express a 55 kDa nuclear protein, which is greatly reduced or undetectable in estrogen receptor negative (ER-) tumors [6]. This observation suggested that this protein may be related to tumor hormonal status and may represent a useful tumor marker. We have carried out preliminary studies to characterize this nuclear protein (referred to as nmt55) from human breast tumors and the MCF-7 cell line, using site-directed monoclonal antibodies and polyclonal antibodies. In this research, we have undertaken biochemical and molecular biology approaches to investigate the function(s) of this protein and its potential role in regulation of human breast cancer cell growth.

Results:

A. Interactions of nmt55 with DNA

Examination of the protein primary structure suggested that nmt55 may contain a helix-turn-helix domain, which may be critical to binding to DNA [6]. Although it is not known if this protein binds to DNA, $p54^{nrb}$, a protein with a high degree of homology to nmt55, cloned from HeLa cells, was shown to bind to a specific DNA sequence derived from the murine long terminal repeat intracisternal A-particle proximal enhancer element (IPE) [7, 8]. To investigate potential binding of nmt55 to DNA, we used gel mobility shift assays to determine the putative DNA binding elements for this protein. These studies were carried out using MCF-7 nuclear extracts and confirmed utilizing bacterially expressed, partially purified nmt55 protein. Using homology sequences with other nuclear proteins, we have shown that nmt55 binds to radiolabeled intracisternal A-particle proximal enhancer element (IPE), with specific DNA sequence, ATCATCAGGGAGTGACACGTCCGA. nmt55 bound IPE probe specifically since unlabeled IPE competed for binding of proteins to IPE probe. To further determine that this IPE probe represent a binding site for nmt55 and further characterize sequence requirements for binding, interaction of nmt55 with IPE probe was carried out in the presence of mutated sequences of IPE and with DNA representing the cAMP response elements (CRE). Mutated IPE (mt2), GATCATCAGGGAAATTTACGTCCGA, reduced nmt55 binding to IPE probe. Mutated IPE (mt3), GATCATCAGTTTGTGACACGTCCGA, and CRE, GATCTTCCCCGTGACGTCAACTCGGC, did not compete for IPE probe binding to nmt55. Mutated IPE (mt4), GATCATCAGGGAGTGTTGCGTCCGA, was very effective in displacing IPE probe from nmt55.

Another protein with a high degree of homology to nmt55, murine NonO, has been shown to bind to a specific DNA sequence (Oct 2) [9]. These octomeric motifs have been identified in the promoter and enhancer regions of many genes [9]. We were unable to demonstrate binding of nmt55 with the Oct 2 sequence utilizing the gel mobility shift assay approach. The native (endogenous) cellular DNA sequences, which bind nmt55, are unknown at

present. These data indicate that nmt55 binds a specific IPE DNA sequence but does not bind the Oct 2 DNA sequence and may play a role in gene regulation.

B. Interactions of nmt55 with cellular RNA, *in situ*.

Further examination of the protein primary structure suggested that nmt55 contains a bipartite RNA binding domain, which is critical for binding to RNA [6]. Although it is not known if this protein associates with RNA, its high degree of homology to other RNA binding proteins, such as $p54^{nrb}$ [7], suggests it may specifically bind to RNA. To investigate this possibility, MCF-7 cells were permeablized and then treated with RNase or DNase to solubilize nmt55. The cells were then washed, and the nmt55 was detected with specific anti-nmt55 antibodies using immunofluorescent conjugated-secondary antibodies. Control experiments utilized fluorescent-labeled antibodies raised against an insoluble, nuclear vault protein. In untreated-control experiments, both nmt55 and vault proteins were detected. DNase treatment, which solubilizes DNA and DNA associated proteins, did not alter the immunofluorescence pattern compared to control as both nmt55 and vault proteins were detected. In contrast, RNase treatment, which solubilizes RNA and RNA associated proteins, solubilized nmt55 but not the vault protein as shown by loss of immunofluorescence staining with anti-nmt55 antibodies. These data indicate binding of nmt55 to RNA, *in situ*.

C. Direct interaction of nmt55 with pre-RNA, *in vitro*.

To investigate the direct binding of nmt55 to RNA, we used gel mobility shift assays. Two separate RNA transcripts were utilized, an actin mRNA (containing only exons) and a β-globin pre-mRNA (containing both introns and exons). As with the DNA gel mobility shift assay studies, these studies were carried out using MCF-7 nuclear extracts and confirmed utilizing bacterially expressed, partially purified nmt55 protein. nmt55 protein from MCF-7 nuclear extracts was shown to bind to the β-globin pre-mRNA but was unable to bind to the actin mRNA. To determine specific binding, we pre-incubated MCF-7 nuclear extract with nmt55 specific antibodies which inhibited nmt55/RNA interaction. Interestingly, bacterially expressed,

partially purified nmt55 protein was unable to bind either transcript. These data suggest two separate findings. First, that nmt55 may not bind RNA directly or may require other proteins to bind RNA. Second, that nmt55 may only bind pre-mRNA and not spliced RNA transcripts. Thus nmt55 may require nucleotide sequences present only in pre-mRNA transcripts.

D. Intermolecular Association of nmt55 with the Polypyrimidine Tract Associated Splicing Factor (PSF) and Topoisomerase I.

The predicted nmt55 amino acid sequence suggested that nmt55 contains a proline and glutamine rich region, previously known to bind other proteins, a bipartite RNA binding domain and possesses strong homology to RNA binding proteins [6-10]. These features indicated that nmt55 may interact with other potential RNA binding proteins and may be involved in RNA processing.

To test this premise, we carried out experiments in which MCF-7 cells were labeled, *in situ*, with ^{35}S methionine, extracted and the total extracts were immunoprecipitated with monoclonal and polyclonal antibodies, directed to specific domains of nmt55. Incubation of total cellular extracts with monoclonal antibody NMT1 (raised against the carboxyl terminus of nmt55), polyclonal antibody NMT5 (raised against a unique peptide in a mid-region of nmt55) or polyclonal antibody NMT4 (raised against a unique peptide in the amino terminus of nmt55) resulted in immunoprecipitation of labeled protein bands at 100 and 55 kDa, respectively. In contrast, incubation of cellular extract with pre-immune serum or unrelated monoclonal antibody, raised against ER, did not result in immunoprecipitation of these specific proteins. These results suggest the specific interaction of nmt55 with a 100 kDa protein.

Since nmt55 shares considerable homology with the RNA binding protein, $p54^{nrb}$ [7], we searched the literature and protein databases to identify any known proteins with an approximate molecular weight of 100 kDa, which may play a role in RNA binding or RNA metabolism. Two proteins were identified. The first protein, termed the polypyrimidine tract-binding protein

associated splicing factor (PSF), was identified by Patton et al. [10] as an essential RNA splicing factor [11] and the second identified protein was Topoisomerase I. It has been demonstrated that Topoisomerase I may have intrinsic kinase activity and may phosphorylate various RNA splicing proteins [12].

To confirm this finding, MCF-7 nuclear extracts were immunoprecipitated with monoclonal antibody NMT1 to immunoprecipitate nmt55 and associated proteins. The precipitates were electrophoresed and immunoblotted using specific antibodies to either nmt55, Topoisomerase I or PSF. Antibodies raised against PSF and Topoisomerase I detected a specific 100 kDa band in the co-immunoprecipitate suggesting that nmt55 is associated with PSF and Topisomerase I. To further substantiate this observation, we carried out immunoprecipitation of MCF-7 nuclear extracts with antibodies raised against PSF or Topoisomerase I and immunoblotted with antibodies raised against nmt55. The data obtained showed that antibodies raised against nmt55 protein immunodetected nmt55 in the co-immunoprecipitate. These observations indicate that nmt55 associates with PSF and Topoisomerase I and that nmt55 may play an important role in regulation of RNA processing and cellular function.

E. Association of nmt55 with Several Essential pre-mRNA Splicing Factors.

The splicing and/or alternative splicing of pre-mRNAs is a critical regulatory step and, as a result, high efficiency of this step is essential in mammalian biology [13, 14]. It has been reported that as many as fifty different proteins may play critical roles in the splicing of pre-mRNAs [11]. Since PSF has been characterized as an essential splicing factor [10] through binding to the polypyrimidine tract in pre-mRNAs, we investigated proteins that interact with the other essential pre-mRNA domains (the 5' splice site, branch A point and 3' splice site) for their ability to associate with nmt55. Reports indicate that several factors have been characterized and their functions partially elucidated [15]. The SR (serine and arginine rich) family of proteins has been shown to have a critical role in pre-mRNA splicing [16, 17]. These proteins are involved in several RNA binding interactions involving all three critical pre-mRNA domains [18]. Inhibition of these proteins has been shown to prevent *in vitro* pre-mRNA splicing [15-18]. U1A 70K protein has been shown to interact with the U1snRNP and aid in its association with the 5' splice

site on the pre-mRNA [19]. U2AF^{65} and U2AF^{35} proteins are critical for the recruitment of the U2 snRNP to the branch A site and 3' splice site on the pre-mRNA, respectively [20, 21].

We investigated the ability of nmt55 to interact with members of the SR, U1 and U2 family using a mAb (16H3) raised against a series of serine and arginine residues [22]. Utilizing immunoprecipitation and Western blot analyses with mAb 16H3, we demonstrated that nmt55 interacts with a series of proteins in a large multi-protein complex. Several members of the SR family interact with nmt55 including SRp75, SRp55, SRp40 and SRp35. nmt55 also associates with U1 70K, U2AF^{65}, U2AF^{35} and Topoisomerase I. Topoisomerase I has been shown to phosphorylate members of the SR protein family and may be important for RNA processing [12]. The association of nmt55 with several well characterized and essential splicing factors strongly suggests a functional role for nmt55 in pre-mRNA splicing.

F. Detection of nmt55 Variants in Human Breast Tumors Using Western Blot and Immunohistochemical Analyses.

As described above, to further our investigation of nmt55 using molecular and biochemical approaches, our laboratory generated two epitope specific anti-peptide polyclonal antibodies (pAbs) to different domains of nmt55 to investigate the role of these domains in nmt55 function. One polyclonal antibody (pAb), termed NMT4, was raised against a unique peptide in the amino terminus of nmt55 and the second pAb, NMT5, was raised against a unique peptide in the mid-region of the protein. These antibodies, along with the monoclonal antibody (mAb) NMT1 (raised against the far carboxyl terminus of nmt55), were then used to screen various human breast tumors using Western blot or Immunohistochemical analyses.

When a series of tumors were screened via Western blot, the pAbs (NMT4 and NMT5) confirmed the original data observed with mAb NMT1 [6] indicating that ER- tumors displayed decreased nmt55 protein expression as compared to ER+ tumors. Interestingly, a subset of ER+ tumors expressed nmt55 protein that was not detected by pAb NMT4 but was detected by NMT5 and NMT1 antibodies using Western blot analyses.

To determine if this observation was the result of molecular changes within the tumor that altered nmt55 protein or rather an artifact of experimental preparation of breast tissue, we used the same set of antibodies to screen paraffin embedded human breast tumors by immunohistochemistry. As was observed with Western blot analysis, a subset of tumors analyzed by immunohistochemistry expressed nmt55 protein, which was detectable only with pAb NMT5 and not with pAb NMT4. Since immunohistochemistry is a powerful tool used to investigate many proteins involved in breast cancer progression and is representative of the *in vivo* tumor environment, we suggest that nmt55 is indeed expressed as a variant, with alterations in its amino terminal domain, in a subset of human breast tumors. This alteration in the amino terminus of nmt55 may have deleterious effects with respect to nmt55 function and thus may play a role in breast cancer progression.

G. Domain Specific Interactions Between nmt55 and PSF.

Since evidence suggested that nmt55 is also expressed in a variant form, we wanted to investigate the domain specific interaction of nmt55 with PSF. To implement in this study, we generated three GST-nmt55 fusion protein constructs. The first construct (GST-FL) encompassed the full-length nmt55 protein (residues 1-471) fused with GST protein, the second construct (GST-NT) fused the amino terminal portion of nmt55 protein (residues 1-227) with GST protein and the final construct (GST-CT) fused the carboxyl terminal portion of nmt55 protein (residues 227-471) with GST protein. These constructs along with GST protein alone were incubated with MCF-7 cell nuclear extracts and subjected to GST pull-down assays and Western blot analysis. Using a specific PSF mAb, we showed that PSF interacts with both the amino terminus and carboxyl terminus of nmt55.

To determine if the nmt55/PSF interaction was specific for these two proteins alone, indicating multiple protein interactions, or rather that these proteins exist in a complex with other, as yet unidentified, proteins, we incubated our GST-nmt55 constructs with purified PSF protein and observed similar results. These results did not eliminate the possibility that nmt55 and PSF are part of a multi-protein complex but suggested that, regardless of other associated proteins, nmt55 and PSF have multiple molecular contacts. Thus, disruption of these contacts or

alteration in domains responsible for these interactions may result in the loss of specific protein function.

H. Role of nmt55 in pre-mRNA splicing and spliceosome formation.

We have also attempted to investigate the specific, functional role of nmt55 in pre-mRNA splicing. To this end, we have designed two approaches. The first approach utilizes a mammalian in vitro splicing assay. In this approach, a purified, radiolabeled β-globin pre-mRNA is spliced in an MCF-7 or HeLa cell extract containing nmt55 protein and is separated into its components using gel electrophoresis. We have been able to produce an efficiently spliced β-globin RNA utilizing this technique. To determine, the specific role of nmt55 in this process we preincubated MCF-7 or HeLa cell splicing extracts with nmt55 specific antibodies. These antibodies are anticipated to prevent nmt55 from interacting with the RNA transcript and other proteins, as described in other assays above. Further, this should specifically identify the step or steps in which nmt55 participates. However, preliminary studies have prevented the initial step of pre-mRNA splicing. Further studies will be required to elucidate this specific function of nmt55 in pre-mRNA splicing.

The second approach was to characterize the role of nmt55 in spliceosome formation. Spliceosome formation is the key initial step in pre-mRNA splicing. Similar to the first approach, radiolabeled β-globin pre-mRNA is incubated in an MCF-7 or HeLa cell extract containing nmt55 protein but for a duration only to allow spliceosome formation but not a complete splice reaction. The spliceosome complex associated with the radiolabeled β-globin pre-mRNA is then separated by centrifugation on a sucrose density gradient. To determine the specific role of nmt55 in this process, we again preincubated MCF-7 or HeLa cell splicing extracts with nmt55 specific antibodies to interrupt nmt55 associations and prevent spliceosome formation. As with the first approach, this should specifically identify the step or steps of spliceosome assembly in which nmt55 participates. Preliminary studies have been promising but further studies are necessary to clearly identify the role of nmt55 in spliceosome formation.

Summary and Significance of the Studies:

In summary, we have identified and characterized a 55 kDa nuclear protein (nmt55) from human breast tumors and the MCF-7 human adenocarcinoma breast cell line using site-directed monoclonal antibodies. We have demonstrated that nmt55 binds RNA and associates with several proteins which are essential for pre-mRNA splicing including the Polypyrimidine tract-binding protein associated splicing factor (PSF), U1 70K, $U2AF^{65}$, $U2AF^{35}$, Topoisomerase I and several members of the SR protein family in human breast tumors and MCF-7 cells. We have also shown that nmt55 protein expression is greatly reduced in ER- human breast tumors and nmt55 protein is expressed in a variant isoform in a subset of ER+ tumors. These data suggest that nmt55 has a potential role as a splicing factor and may be critical to cell growth and function. Thus, decreased nmt55 expression in ER- human breast tumors or nmt55 post-transcriptional variants in ER+ tumors may indicate loss of normal growth. nmt55 may be involved in pre-mRNA processing of ER, and loss of its expression in tumor cells may result in loss of ER expression due to lack of proper pre-mRNA splicing. The decreased expression of nmt55 may be related to tumor differentiation, unregulated tumor cell growth and metastases and may explain tumor heterogeneity, especially with respect to hormonal interventions in the course of treatment. In this study, we identify, for the first time, a protein with potential involvement in pre-mRNA splicing which may be an important tumor marker for breast cancer progression. Further investigation of the functional relationhsip between nmt55 and breast cancer progression may provide critical information needed for early detection of potentially metastatic tumors, improve diagnosis and prognosis and aid in developing strategies for therapeutic management and care of breast cancer patients.

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Appendix

Key Research Accomplishments

- We have identified the interaction between nmt55 and Polypyrimidine Tract Binding Protein Associated Splicing Factor (PSF), an essential mammalian splicing protein, using co-immunoprecipitation experiments.

- We have determined that nmt55 has the ability to bind specific DNA sequences, such as the Intracisternal A-particle proximal enhancer element (IPE), in vitro using an electrophoretic mobility shift assay (EMSA) approach.

- We have demonstrated the specific binding of nmt55 to pre-mRNA, in vitro, using an electrophoretic mobility shift assay (EMSA) approach. Further, we have determined that nmt55 preferentially binds cellular RNA over DNA, in situ, utilizing solubility experiments.

- We have detected nmt55 protein variants in a subset of human breast tumors. These nmt55 protein variants have alterations in their amino terminal region as determined by Western blot and immunohistochemical analyses using an epitope specific anti-peptide polyclonal antibody raised against the amino terminus of nmt55.

- We have determined the nmt55 domains responsible for protein interaction with the Polypyrimidine Tract Binding Protein Associated Splicing Factor (PSF). It was determined using GST-nmt55 fusion proteins and GST Pull Down Assays that nmt55 has multiple molecular contacts with PSF.

- We have identified a series of essential splicing factors (PSF, U1 70K, U2AF65, U2AF35, SRp75, SRp55, SRp40, SRp35 and Topoisomerase I) that associate with nmt55 in a multi-protein complex in human breast tumors and cell lines. These associations were determined by co-immunoprecipitation experiments coupled with Western blot analysis utilizing specific monoclonal and polyclonal antibodies.

Reportable Outcomes

Degree Obtained:

Doctorate of Philosophy (Ph.D.) in Biochemistry; Boston University School of Medicine; May 2001

Publications:

Pavao M and Traish AM. "Estrogen receptor antibodies: specificity and utility in detection, localization and analyses of estrogen receptor alpha and beta." Steroids 66(1):1-16, 2001.

Pavao M, Huang YH, Hafer LJ, Moreland RB and Traish AM "Immunodetection of nmt55/p54nrb Isoforms in Human Breast Cancer." Submitted

Pavao M., Moreland RB, Schmitt H, Tucker PW and Traish AM "Interaction of nmt55/p54^{nrb} with Essential Splicing Factors and pre-mRNA in Spliceosome Formation." Manuscript in Preparation

Published Abstracts:

Pavao M, Moreland RB and Traish AM. "Potential Role of a Novel Nuclear Protein (nmt55) in Human Breast Cancer." Abstract, The FASEB Journal, 2000.

Presentations:

Pavao M, Moreland RB and Traish AM. "Identification and Characterization of a Novel Nuclear Protein (nmt55) in Human Breast Cancer." Henry I Russek Student Achievement Day. May 2001 (Boston, MA).

Pavao M, Moreland RB and Traish AM. "Characterization of nmt55 as a Potential Tumor Marker in Human Breast Cancer." Graduate Student Science Research Day. April 2001 (Boston, MA).

Pavao M, Moreland RB and Traish AM. "Characterization of a Novel Nuclear Protein (nmt55) in Human Breast Cancer." Era of Hope: Department of Defense Breast Cancer Research Program. June 2000 (Atlanta, GA).

Pavao M, Moreland RB and Traish AM. "Potential Role of a Novel Nuclear Protein (nmt55) in Human Breast Cancer." American Society for Biochemistry and Molecular Biology. June 2000 (Boston, MA).

Pavao M, Moreland RB and Traish AM. "Identification and Characterization of a Novel Nuclear Protein (nmt55) in Human Breast Cancer." Henry I Russek Student Achievement Day. May 2000 (Boston, MA).

Pavao M, Moreland RB and Traish AM. "Characterization of nmt55 as a Potential Tumor Marker in Human Breast Cancer." Graduate Student Science Research Day. April 2000 (Boston, MA).

Pavao M, Moreland RB and Traish AM. "Potential Role of nmt55, a Novel Nuclear Protein, as a Tumor Marker in Human Breast Cancer Progression and Metastasis." Gordon Research Conference on Hormonal Carcinogenesis August 1999 (Tilton, NH)

Personnel:

Matthew Pavao, Ph.D.

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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 26 Aug 02

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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